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Bibliography.

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Summary.

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(57) [Abstract] (******)
[Objects of the Invention] The nucleobase sequencing method that the base sequence of a nucleic acid can be determined in a short time is offered.
[Elements of the Invention] The oligonucleotide which includes the array of all k bases as a primer is fixed in each column. And after adding strange ******-ed [single strand] in each column of the capillary-tube plate CA with which it did in this way and the primer was fixed in each column and making the primer and ******-ed in each column hybridize, DNA polymerase is simultaneously added for four kinds of dNTP(s) to each column as a catalyst. In the direction of 3', a base complementary to each base of ******-ed hybridizes with the base of ******-ed from 5' of a primer by this, and the 1 direction extension reaction of a primer arises. By measuring this amount of extension, the joint position of the united primer and the united primer, and ******-ed is identified.

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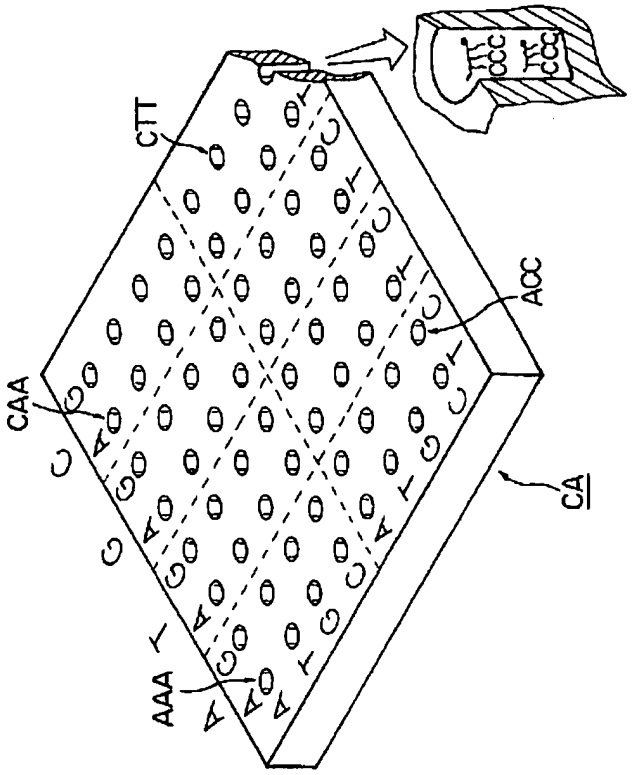

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CLAIMS

[Claim(s)]
[Claim 1] The base-sequence-determination method of ******-ed of the single chain characterized by providing the following. The 1st process which forms the complex which is made to carry out hybridization of the primer (oligonucleotide) and ******-ed of a single chain which have been arranged by separating into two or more fields for every kind, and has the double chain portions of mold and a primer. the above -- plurality -- a field -- arranging -- having had -- each -- the above -- complex -- the above -- complex -- mold -- a nucleotide -- complementary -- base pairing -- carrying out -- obtaining -- two or more -- a kind -- a nucleotide -- or -- a nucleotide analog -- substantial -- simultaneous -- in addition -- the above -- -ed -- ****** -- mold -- having carried out -- a primer -- five -- ' -- from -- three -- '

-- a direction -- extension -- a reaction -- carrying out -- the -- two
[Claim 2] The nucleobase sequencing method according to claim 1 characterized by what is prescribed by the column of the capillary-tube plate with which two or more aforementioned fields consist of material made from glass or silicon.
[Claim 3] The nucleobase sequencing method according to claim 1 characterized by what the pyrophosphoric acid isolated from two or more aforementioned kinds of nucleotides or the nucleotide analog with the extension reaction in the 2nd process of the above in the amount of extension of the primer in the 3rd process of the above is made to react with APS, ATP is generated, and is performed by detecting the amount of luminescence accompanying the luciferase reaction of this ATP.
[Claim 4] The extension reaction of the primer which added the deoxyribonucleotide 3 phosphoric acid (dNTP) which carried out the fluorescence label as two or more kinds of nucleotide analogs in the 2nd process of the above, and used ******-ed as mold is performed. The nucleobase sequencing method according to claim 1 characterized by what is performed by detecting the amount of luminescence from the fluorescence label of the primer which irradiated excitation light and elongated the amount of extension of the primer in the 3rd process of the above to two or more aforementioned fields.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] this invention relates to the nucleobase sequencing method which can acquire a living body's gene information by analyzing a nucleic acid in the living body by physical and chemical technique.
[0002]
[Description of the Prior Art] As for the base sequence of DNA, two, Sanger's and others dideoxy chain terminator method (5467 Sanger, F., Nicklen, S.and Coulson, .A.R., Proc.Natl.Acad.Sci.USA 74:5463- 1977) and the chemical decomposition method (564 W. Maxam, A.M.and Glibert, Proc.Natl.Acad.Sci.USA 74:560- 1977) of Maxam-Glibert, are put in practical use. After carrying out a label, making and compounding or decomposing by radioisotope, the fluorochrome, etc. so that all can identify the kind

of end base of the fragment of various length of ** DNA-ed, by carrying out gel electrophoresis, each fragment is divided into short order and an array is guessed by reading the kind of base of an end in an order from the shorter one, and going. Although these methods were improved and it had continued till present, since electrophoresis had separated all, great time and a great effort had been required. In order to determine a large-scale base sequence like all people's GUNOMU (about 3 billion base sequences), the more efficient new method is searched for.

[0003] It is sequencing as one of such the new methods. by What is called hybrization (SBH) method is advocated (128 G.C. J.Theor.Biol. 135:303 -307, 1988; Bains, Wand Smith, Darmance, R., Labat, I., Brukner, I.and Crkvenjakov, R., Genomics 4:114- 1989).

[0004] The foundations of this method are the composition FK of all the oligonucleotides of the length k base contained in it in the base sequence of ** DNA-ed. It is in making a shell alignment decision. The algorithm of alignment determination can result in the problem of the picture drawn without lifting the brush from the paper of the figure often seen with a puzzle (73 P. Pevzner, A.J.Biomol.Struct.Dyn.7:63-1989). Pevzner and others (1991) is finding the length of DNA which can be determined by 95% of probability based on oligonucleotide composition of a length k base by the simulation (419 K R.Belyavskii, A.V., Florent Pevzner, P.A., Lysov, Y.P., Khrapko, ev, V.L. and Mirzabekov, A., and J.Biomol.Struct.Dyn. 9:399- 1991). For example, if the oligonucleotide of length 8 base which has 65536 kinds is used, as shown in drawing 10 , an alignment decision of the DNA sequence of about 180 bases will be made. With the actual determining method, it is composition FK by hybridization (hybridization) with the oligonucleotide of k base about the single strand of ******-ed. It finds out. Therefore, ******-ed to which the number of bases exceeds about 180 by ******-ed cannot be made to cross in the case of eight bases. Moreover, since an oligonucleotide has no less than 65536 kinds in this case, in order to constitute practical equipment, it is necessary to fix an oligonucleotide or ******-ed in the shape of [of the size of the grade which can be set to a measuring instrument] a matrix (this method is called sequencing by hybrization with oligonucleotide matrix (SHOM) method.).

[0005]

[Problem(s) to be Solved by the Invention] However, it is pointed out that there are two big troubles in the SBH/SHOM method explained above (657 a Suyama Ming protein nucleic-acid enzyme, 38:647- 1993). One is the problem of the mismatch by imperfect hybridization and partial hybridization. If partially complementary even if not completely complementary, an oligonucleotide can be crossed with ******-ed and may be included in alignment calculation to the array which originally is not included in ******-ed. Another is the problem of a repeat array (or the same nucleotide sequence). Although it turns out that the base sequence is contained only by detecting hybridization when two or more same nucleotide sequences as ******-ed are contained, it becomes very difficult to determine each position of a base sequence repeatedly only by alignment by the computational algorithm.

[0006] Furthermore, although there is no repeat array, it cannot determine this base sequence uniquely by the SBH method. The easy example for below is shown.

[0007] First, ******-ed presupposes that it is [ATGAT]. In this case, supposing the

primer of 3mer hybridizes, the partial array of this ******-ed will be three, [ATG], [TGA], and [GAT]. However, when it is going to determine the original array by aligning these three partial arrays, it is in the base sequence of ******-ed.

(場合1)

```
          ATG
           TGA
            GAT
```

(場合2)

```
              GAT
               ATG
                TGA
****
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The case of two kinds of ** can be considered. therefore, by the SBH method, the array of a nucleobase cannot be determined uniquely -- things -- **

[0008] and although the SBH/SHOM method also looks a twist like [the sequencing method before these methods] markedly and the processing time is shortened, to be able to determine the base sequence of these nucleic acids further in a gene analysis in a short time is desired

[0009] this invention is made in view of such a problem, and sets it as the main purpose to offer the nucleobase sequencing method which can determine the base sequence of a nucleic acid quickly.

[0010]

[Means for Solving the Problem] In order to solve the above problem, this invention is set to the base-sequence-determination method of ******-ed of a single chain for the nucleobase sequencing method of a single chain. The 1st process which forms the complex which is made to carry out hybridization of the primer (oligonucleotide) and ******-ed of a single chain which have been arranged by separating into two or more fields for every kind, and has the double chain portions of mold and a primer, Two or more kinds of nucleotides or the nucleotide analog which it is complementary to the nucleotide of the mold of complex, and can carry out base pairing to each complex arranged to two or more fields at it is added simultaneously substantially. We decided to include the 2nd process which performs the extension reaction from 5' of the primer which used ******-ed as mold to the direction of 3', and the 3rd process which detects the amount which the primer elongated.

[0011]

[Function] According to the nucleobase sequencing method concerning this invention, first thus, according to the 1st process Carry out hybridization of the primer and ******-ed which have been arranged by separating into two or more fields for every kind, and these complex is formed. next -- the -- two -- a process -- -ed -- ****** -- mold -- ****** -- this -- involution -- carrying out -- obtaining -- a nucleotide -- or -- a nucleotide analog -- substantial -- simultaneous -- in addition -- at once -- -ed -- ****** -- mold -- having carried out -- a primer -- five -- ' -- from -- three -- ' -- a direction -- extension -- a reaction -- carrying out . And the 3rd process detects the amount of extension of these primers. Since the amount of extension of each primer arranged to two or more fields, i.e., the joint position of a

primer and ******-ed, becomes clear by this and the kinds of primer in each field differ, if the base sequence of a primer is analyzed using the so-called algorithm of a picture drawn without lifting the brush from the paper, it is possible to determine the base sequence of ******-ed.

[0012] Moreover, the primers which such two or more fields' being specified by the column of the capillary-tube plate which consists of material made from glass or silicon, then the column were separated, and have been arranged to another field since it was mutually independent are not mixed. Although the column has arranged in the shape of a matrix from a viewpoint of sequencing of ******-ed and the shape's of a rectangle is useful as such a capillary-tube plate configuration, the thing of a round shape or a triangle is also applied as this configuration, and it sells at this invention. Moreover, since it is generally prescribed by the number of diameters, or the about hundreds of micrometers cylindrical bore, in case the column of such a capillary-tube plate introduces liquids, such as a primer and ******-ed, in a column, it is desirable [a column] to use the electrophoresis to the thickness direction of a capillary-tube plate in consideration of surface tension. Furthermore, it is more desirable to fix a primer in the column of a capillary-tube plate from a viewpoint of sequencing of ******-ed.

[0013] Moreover, although various things can be considered to the method of detection of such an amount of extension, it is good also as carrying out by, making the pyrophosphoric acid isolated from two or more kinds of nucleotides, or the nucleotide analog with the extension reaction in the 2nd process in the amount of extension of the primer in the 3rd process of the above react with APS for example, generating ATP, and detecting the amount of luminescence accompanying the luciferase reaction of this ATP. It is possible to ask for the joint position of the length, the primer, and ******-ed which were elongated by this since the amount of luminescence was proportional to the amount of extension.

[0014] Moreover, it is good also as performing the extension reaction of the primer which added the deoxyribonucleotide 3 phosphoric acid (dNTP) which carried out the fluorescence label as two or more kinds of nucleotide analogs in the 2nd process, and used ******-ed as mold, and carrying out by detecting the amount of luminescence from the fluorescence label of the primer which irradiated excitation light and elongated the amount of extension of the primer in the 3rd process to two or more fields. It is possible to ask for the joint position of the length, the primer, and ******-ed which were elongated by this, since the amount of luminescence observed by irradiation of excitation light from each complex is proportional to the amount of extension.

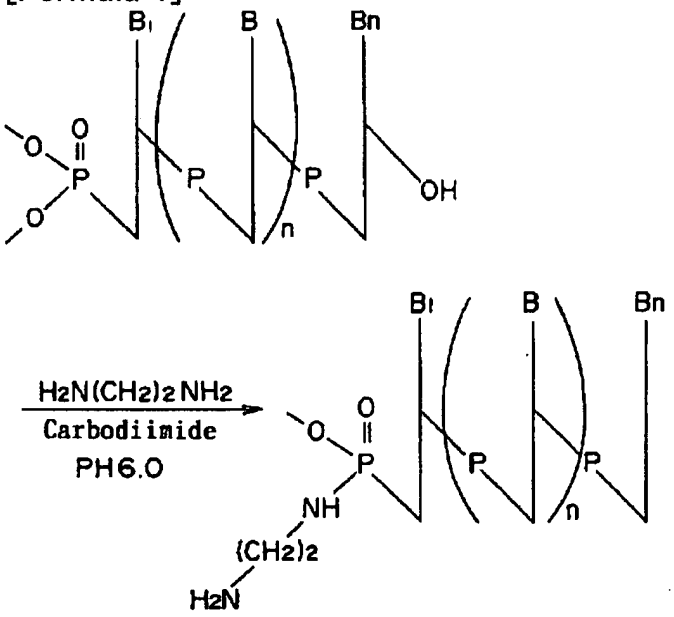
[0015]

[Example]

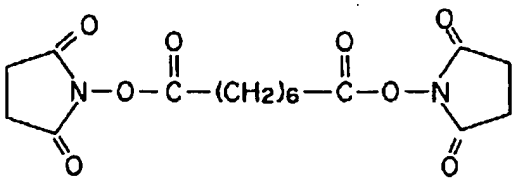
(The 1st example)

(1) Carry out easy [of the glass capillary-tube plate CA of the rectangular board which has two or more columns as shown in drawing 1 first], and fix the oligonucleotide which includes the array of all k bases, 3 [in this drawing] of all the combination of A, T, G, and C mer(s), i.e., 64 columns, as a primer like the SBH/SHOM method in each column.

[0016] (a) These oligomer is applied. It is producible using automatic composition machines, such as a 381A type machine by the biotechnology system company.
[0017] (b) and fixation of the primer which carried out in this way and was compounded -- as follows -- carrying out -- a line -- things are made
[0018] ** Fix an AMINO (-NH2) machine in each column. First, a mask is given to portions other than the column of the capillary-tube plate CA, and the capillary-tube plate CA in which this mask was formed is installed in airtight containers, such as a desiccator, at this fixation. After an appropriate time, aminopropyl trimethoxysilane is made full [gasify and] in this airtight container, and it is left for several hours. Then, BEKU [this capillary-tube plate CA / with oven (BEKU furnace) / about 160 degrees C].
[0019] ** Attach an AMINO (-NH2) machine to 5'edge of the primer compounded by the automatic synthesizer unit next. This is H2 N(CH2)2 NH2 to a primer. NH-(CH2) 2-H2 N is fixable to 5'edge by making carbodiimide (carbodiimide) (pH 6) react.
[0020] That is, this reaction follows the following reaction formulae.
[0021]
[Formula 1]

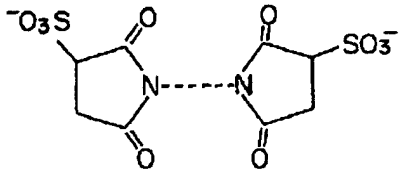


[0022] ** BAIFANKUSHONARU cross linker [(disuccinimidyl suberate), for example, JISAKUSHIMI dill SABERATO, which carries out covalent bond to an AMINO (-NH2) machine after an appropriate time
[0023]
[Formula 2]

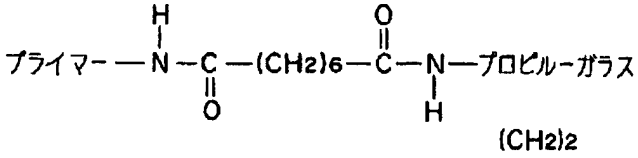


[0024] Or water-soluble homolog screw SARUFUSAKUSHINIMI dill of this SABERATO(bis-sulfusuccinimidyl suberate)]

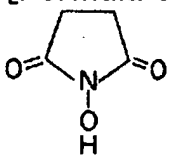
[0025]
[Formula 3]



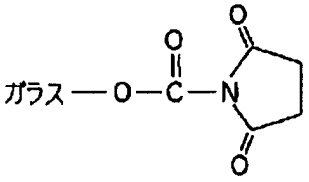
[0026] It is [0027] when it adds to ******.
[Formula 4]



[0028] It ******.
[0029] However, since there is a possibility that primers may join together when such a reaction is used, a COOH basis is attached to the amino group which attached the COOH basis to the aminopropyl TORIMEKI lion run, or was fixed to the column (glass), and a glass-COOH basis is formed into a SAKUSHINIMI dill as mentioned above.
[0030] It is here and is N-hydro KISHISAKUSHINIMI dill [0031].
[Formula 5]



[0032] It is [0033] when come out and activated.
[Formula 6]



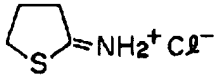
[0034] It ******. If the amination primer obtained by this by ** is mixed, these will carry out a crosslink.
[0035] Moreover, it is glass by what time difference is attached and added to a BAIFANKUSHONARU cross linker and an amino primer for (that is, an amino primer is first added to a glass-linker). -(linker)- A primer can be obtained. Namely, [0036]
[Formula 7]

ガラス-リンカー
↓
ガラス-リンカー+アミノプライマー
↓
ガラス-リンカー+プライマー

[0037] It can carry out and a primer can be efficiently fixed to glass.
[0038] Furthermore, a primer is also efficiently fixable to (- sulfhydryl group) of each column using the procedure of the following ** - **.

[0039] ** First, make mercapto propyltrimethoxysilane react on all column front faces, and fix (- sulfhydryl group). In addition, this fixation is the reagent [0040] of Traut to the aforementioned column-amino group.

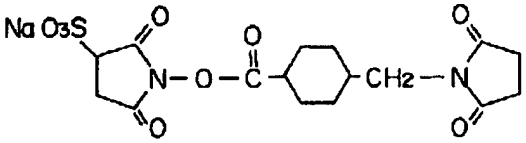
[Formula 8]



[0041] it ****** -- making (- sulfhydryl group) -- it can also add

[0042] ** Next, add the amino group to 5'edge of each primer beforehand by the same method as the above, and it is a BAIFANKUSHONARU cross linker there (covalent bond of the sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) is carried out (pH 7).). In addition, the structure expression of this is [0043].

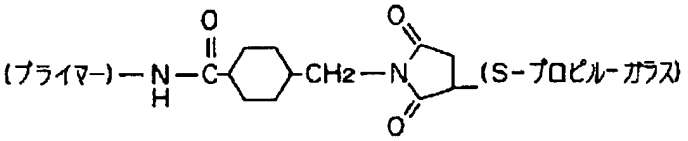
[Formula 9]



[0044] It comes out.

[0045] ** The primer which carried out in this way and was activated is [0046], supposing it introduces a separate primer into each column (pH 6), since it reacts with (- sulfhydryl group) and covalent bond is carried out.

[Formula 10]



[0047] ** -- it is fixed like

[0048] In addition, in the column of the capillary-tube plate CA of drawing 1 , by the primer having arranged by the oligonucleotide composition shown in drawing 2 , pouring into the column of a primer makes electric field produce in the thickness direction of the capillary-tube plate CA, and electrophoresis performs it while it penetrates the primer pouring needle from an automatic synthesizer unit in a column in consideration of the surface tension of the liquid (primer) to pour in. An oligonucleotide will be fixed in a column so that a part may be extracted, and it may expand and may be typically shown among drawing 1 , as a result of following the above-mentioned procedure (all over this drawing, a primer is [CCC]).

[0049] (2) And add strange ******-ed [single strand] (for example, a base sequence sets to [AGCTGCTA].) in each column of the capillary-tube plate CA with which it did in this way and the primer was fixed in each column (for example, column of [GAC]) (drawing 3 (b)). This hybridizes the primer in each column with ******-ed. (hybridization) It carries out. Then, ******-ed which was not crossed washes and washes away. And in the column, the buffer solution is introduced so that the following polymerase reactions and luciferase reactions may be performed smoothly (drawing 3 (c)). As this buffer solution, the glycerol as a sequence buffer, A glucose,

APS, luciferin, the tris-hydrochloric acid as the pH buffer solution, etc., The complex used as the substrate of a polymerase reaction or a luciferase reaction (it dNTP(s)) NaCl etc. uses the day thio sleigh toll for protecting enzyme protein from oxidization denaturation etc. as salting-in liquid for raising MgCl2 for generating ATP and the complex of Mg2+, and activation of a reaction, and raising stability.

[0050] (3) These ******-ed add DNA polymerase for four kinds of dNTP(s) (namely, dATP, dTTP, dGTP, dCTP) to a primer and each crossed column simultaneously as a catalyst (drawing 3 (d)). In the direction of 3', a complementary base hybridizes with the base of ******-ed from 5' of a primer by this at each base (A; an adenine, T; a thymine, G; a guanine, C; cytosine) of ******-ed, and the 1 direction extension reaction of a primer arises (typically shown in drawing 3 (b)).

[0051] Here ******-ed (for example, [AGCTGTCA]) Since it has crossed with the primer of [TGC], [CGA], [GAC], [ACG], and [GAT], in the column to which [TGC] was fixed According to the following reaction formulae (1), the pyrophosphoric acid (PPi) of 5 separates with an extension reaction, and PPi of n= 2.5, and 3, 2 and 0 separates in the column of [CGA], [GAC], [ACG], and [GAT], respectively.

[0052]

Template primer +ndNTP -> template-(primer +ndNMP)+nPPi -- Reaction formula (1)

(4 In addition, in order to raise the accuracy of measurement since dATP and APS which are contained in system of measurement also serve as a substrate and carry out a luminous reaction a little although the substrates of the luminous reaction by the luciferase are luciferin, ATP, and an enzyme, before these matter performs a luminous reaction, it is necessary to remove it in enzyme.) That is, by adding HEKISONAZE (specifically HEKISONAZE fixed bead) to these columns, Pi is separated from excessive dATP which remained by the morphosis of (1) formula, and it is made dADP (drawing 3 (e)).

[0053] By adding a glycerokinase (specifically glycerokinase fixed bead) to each column, Pi is isolated from dATP which remains in a column according to the following reaction formulae (2).

[0054]

dATP->dADP+Pi -- Reaction formula (2)

(5) Next, by adding an ATP sulfurylase to each column, PPi in a column is made to react with APS according to the following reaction formulae (3), and it is referred to as ATP (drawing 3 (f)).

[0055]

PPi+APS->ATP -- Reaction formula (3)

In addition, APS which remained in the column adds AP sulfatase to a column, and decomposes into it.

[0056] (6) Arrange after this in the black box DB of a nucleobase sequenator as shows the capillary-tube plate CA to drawing 4 . In addition, the black box DB which the nucleobase sequenator shown in drawing 4 contains the capillary-tube plate CA, and intercepts the light from the outside, The microscope or micro lens 20 which is prepared in the unilateral side of a black box DB, and can observe the inside of the column of the capillary-tube plate CA, The CCD television camera 10 which picturizes

the capillary-tube plate CA observed from a microscope or a micro lens 20, The image processing system 30 which carries out the image processing of the video signal from this CCD camera 10 (for example, the background lightwave signal of the intensity below a threshold is removed by letting a filter pass), Based on the signal from an image processing system 30, it consists of computers 40 which determine the array of ******-ed with the algorithm of a picture drawn without lifting the brush from the paper. After arranging the capillary-tube plate CA in such a nucleobase sequenator, (drawing 3 (g)) and luminescence which is proportional to the amount of ATP according to the following reaction formulae (luciferase reaction) (4) are obtained by adding a luciferase (specifically luciferase fixed bead) to a column (drawing 3 (h)).

[0057]

Luciferin +ATP+O2 -> oxy-luciferin +AMP+PPi+CO2+ light -- Reaction formula (4)

That is, since the amount of ATP here is proportional to the amount of PPi isolated from ******-ed, the amount of PPi is proportional to the amount of the base which carried out the extension reaction and a primer is not elongated in the direction of 3'edge from 5'edge, the existence [primer / ******-ed and] of hybridization and the joint position of ******-ed and a primer are known. If this capillary-tube plate CA is observed with the nucleobase sequenator shown in drawing 4 , luminescence as shown in drawing 5 (a) will be observed. In addition, it is as the primer in the column of the capillary-tube plate CA having also shown the array to drawing 2 as mentioned above.

[0058] That is, in [TGC], [CGA], [GAC], [ACG], and the [GAT] column, since luminescence of the amount 5 of luminescence, 2.5, and 3, 2 and 0 is observed, respectively, if these are arranged in order with few amounts of luminescence according to the algorithm of a picture drawn without lifting the brush from the paper, the base sequence elongated to a primer called TCGACGAT will be determined. And since ******-ed is a nucleic acid complementary to this, ******-ed can be identified [AGCTGCTA] (drawing 5 (b).).

[0059] Thus, if it carries out based on the length of the measured primer, the mutual physical relationship of each oligonucleotide will be searched for within the limits of the accuracy of measurement. If the accuracy of measurement improves and the difference below k base can be detected, as shown in drawing 6 , the base sequence of ******-ed can be determined in this stage. This drawing expresses the base sequence method of ******-ed by measuring the amount of isolation of the pyrophosphoric acid produced by the method and this primer extension reaction of hybridization at the time of using the primer of 8mer(s) into a column, using 3'-GCCACCTCGAGGTTAAGCGGGATATCACTCAGCATAATCGCCGCGAGTGACCGGCA -5' as ******-ed. Thus, by lengthening the length of the primer in a column, even when ******-ed becomes long-chain according to the method of this example If this nucleobase sequencing method is used, since it is possible to measure not only the kind of primer but the joint position of this and ******-ed of mold, it not only can determine the array of a nucleobase in a short time, but Two or more oiler paths of the picture drawn without lifting the brush from the paper which has not been determined since a joint position was not able to be pinpointed are able to determine the array of a certain nucleobase to some extent.

[0060] In a certain case, the limit of error of the accuracy of measurement should just ask more than k base by the computational algorithm stated by the describing [above] SBH method in the portion which cannot be determined. or [moreover, / fixing an oligonucleotide or ******-ed in the shape of / of the size of the grade which can be set to a measuring instrument / a matrix, in order to measure precisely and simply (for example, 8mer)] -- or compartmentalization is carried out and it can be necessary to be made to perform hybridization (for example, 65536 kinds), extension, and measurement Moreover, if ******-ed or an oligonucleotide is fixed in a tube wall, extension reaction operation will become easy.

[0061] Next, DNA polymerase was actually introduced for four kinds of dNTP(s) (namely, dATP, dTTP, dGTP, dCTP) as a catalyst within the column, and the following experiments confirmed whether luminescence proportional to the joint position of the ******-ed and the primer resulting from isolation of the pyrophosphoric acid accompanying hybridization and this and isolation of a pyrophosphoric acid would take place by adding four kinds of dNTP(s) to ******-ed [abbreviation simultaneous].

[0062] (Example 1 of an experiment) At the extension reaction by the above-mentioned DNA polymerase, if one dNTP is incorporated by DNA, one pyrophosphoric acid will separate. Therefore, the amount of an extension reaction will be calculated if the fixed quantity of the amount of the separating pyrophosphoric acid is carried out. Since the capillary-tube plate which is used for actual base-sequence-determination equipment and which has the compartment of 1 million, for example was equivalent to what bundled the test tube of 1 million, the experiment the above-mentioned invention is proved [experiment] for three test tubes here was conducted, and the amount of luminescence resulting from the pyrophosphoric acid isolated from ******-ed was detected. In addition, since it was unrelated to the phenomenon of luminescence, fixation of a primer was not performed. Here, the amount of a pyrophosphoric acid was transposed to the amount of ATP using an adenosine 5'phospho sulfuric acid (APS) and ATP SARUFURIRAZE, and was detected by the luminous reaction by the luciferase.

[0063] (Material and method)

One reagent glycerokinase fixed sepharose bead hexokinase fixed sepharose bead ATP sulfurylase fixed sepharose bead AP sulfatase fixed sepharose bead luciferase fixed sepharose bead 224 base length chain DNA (multi-cloning site of a plasmid) Reverse primer KS primer DNA polymerase dNTP buffer solution (dATP-dTTP-dGTP-dCTP every 25microM, ten glucose glycerol mM(s) each, APS1micromM, luciferin 100microg/ml, 40mM-tris and a hydrochloric acid (pH 7.5), 20 mM-MgCl2, 50 mM-NaCl, 10mM-day thio sleigh toll)

Here, the base sequence as a reverse primer is [5'-AACAGCTATGACCATG-3']. It is ** and the base sequence of KS primer is [5'-CGAGGTCGACGGTATCG-3']. It is **.

[0064] and as a base sequence of ******-ed [3'-
TTGTCGATACTGGTACTAATGCGGTTCGCGCGTTAATTGGGAGTGATTTCCCTTGTT
GGGAGCTCCAGCTGCCATAGCTATTCGAACTATAGCTTAAGGACGTCGGGCCCCCT
TGGCGCCACCTCGAGGTTAAGCGGGATATCACTCAGCATAATGCGCGCGAGTGACC
-5']

********.
[0065] the object for laboratory instrument thermostat test tubes -- photomultiplier-tube type integrating-sphere universal HOTON counter experiment operation **** with a measurement electrode holder and three test tubes (a-c) were prepared, it was alike, respectively, and the following were added
[0066] a) dNTP buffer solution (100microl)
b) The dNTP buffer solution (100microl), the 1 chain DNA (two picomoles), KS primer (one picomole)
c) The dNTP buffer solution (1ml), the 1 chain DNA (two picomoles), a reverse primer (one picomole)
And it is after [cooling] DNA polymerase (three units were added, it incubated for 5 minutes and 37 degrees C was saved in Hikami.) in Hikami immediately after incubation for 65 degrees C and 3 minutes about each test tube.
[0067] 10micro of enzyme fixed sepharose bead suspension I was added to each test tube in order of following (1-4), it agitated calmly, and top ** was moved to the new test tube after incubation for 25 degrees C and 3 minutes.
[0068] 1. After 10microl Adding the luciferase fixed bead to the glycerokinase fixed SEFARO bead 2. HEKISONAZE fixed sepharose bead 3.ATP sulfurylase fixed sepharose bead 4.AP sulfatase fixed sepharose bead last top ** and agitating calmly, it set to the integrating sphere immediately and the luminescence quantity of light was counted. As a result of measuring the amount of luminescence, it is 0.19x107 in 204.8 seconds after the test tube (a) in which a primer is not contained. With the test tube (b) in which the photoelectron pulse of an individual was measured and the reverse primer is contained, it is 2.06x107. With the test tube (c) in which the photoelectron pulse of an individual was measured and KS primer is contained, it is 1.45x107. The photoelectron pulse of an individual was measured.
[0069] That is, it is expected from the base sequence of ******-ed that the length which reverse and KS primer elongate by hybridization with the above-mentioned ******-ed is 208 and 127, respectively. Therefore, it is thought that the amounts of isolation of the pyrophosphoric acid accompanying this hybridization are 208 and 127, respectively, and it is shown that the above-mentioned experimental result can estimate the length of each primer elongated with the error of the number of about 13 bases. Since it is thought that the error of this number of about 13 bases is based on background luminescence, the work which suppresses background luminescence further is carried out, and if improvement is added to a measuring device, it will be thought that detection precision improves. That is, if this nucleobase sequencing method is used, since it is possible it not only can to determine the kind of primer combined for a short time, but to measure the joint position of this and ******-ed which is mold, two or more oiler paths of the picture drawn without lifting the brush from the paper which has not been determined since a joint position was not able to be pinpointed are able to determine the array of a certain nucleobase to some extent.

[0070] (The 2nd example) In the 1st above-mentioned example, the base sequence of ******-ed was able to be determined by carrying out hybridization of the ******-ed to a primer, and carrying out the fixed quantity of the amount of the pyrophosphoric

acid which separated by this hybridization. Next, how to determine a nucleobase array using the fluorescence label object dNTP is explained.

[0071] (1) First fix the oligonucleotide which includes the array of all k bases, 3 [in this drawing] of all the combination of ATGC mer(s), i.e., 64 columns, in the glass capillary-tube plate CA of the rectangular board which has two or more columns as shown in drawing 1 as a primer in each column like the 1st example (drawing 7 (a)).

[0072] (2) And add strange ******-ed [single strand] (for example, a base sequence sets to [ATCTGCTA].) in each column of the capillary-tube plate CA with which it did in this way and the primer was fixed in each column (for example, column of [GAC]) (drawing 7 (b)). This hybridizes the primer in each column with ******-ed (hybridization). Then, ******-ed which was not crossed washes and washes away. And in the column, the buffer solution is introduced so that the following polymerase reactions and luciferase reactions may be performed smoothly (drawing 7 (c)). Suppose that it is the same as that of the thing of the 1st example as this buffer solution.

[0073] (3) These ******-ed add DNA polymerase for four kinds of fluorescence-dNTP(s) (namely, dATP, dTTP, dGTP, dCTP) to each column which carried out the polymerization to the primer simultaneously as a catalyst (drawing 7 (d)). In the direction of 3', a complementary base hybridizes with the base of ******-ed from 5' of a primer by this at each base (A; an adenine, T; a thymine, G; a guanine, C; cytosine) of ******-ed, and the 1 direction extension reaction of a primer arises (typically shown in drawing 7 (b)). Then, washing removes fluorescence-dNTP from the inside of a column.

[0074] Here ******-ed (for example, [AGCTGTCA]) Since the polymerization is carried out to the primer of [TGC], [CGA], [GAC], [ACG], and [GAT], in the column to which [TGC] was fixed While the fluorochrome of n= 5 is incorporated according to the following reaction formulae (1) with an extension reaction, in the column of [CGA], [GAC], [ACG], [CGA], and [GAT], the fluorochrome of n= 2.5, and 3, 2 and 0 is incorporated, respectively.

[0075] (4) Arrange after this in the black box DB of a nucleobase sequenator as shows the capillary-tube plate CA to drawing 8 . The nucleobase sequenator shown in drawing 8 contained the capillary-tube plate CA, and is equipped with the black box DB1 which intercepts the light from the outside, the excitation light source (for example, xenon lamp) 70 which irradiates excitation light at this capillary-tube plate CA, and the CCD television camera 10 which is prepared in the unilateral side of a black box DB1, and picturizes the column of the capillary-tube plate CA. Moreover, the excitation light-transmission filter 50 is formed between the excitation light source 70 and the capillary-tube plate CA, and it has composition which is made to penetrate only short wavelength (for example, peak value of 450nm) among the wavelength components of the light which penetrates this as shown in this drawing (c), and irradiates the capillary-tube plate CA. Moreover, the fluorescence transparency filter 60 is installed between the capillary-tube plate CA and the CCD television camera 10, and the light more than fluorescence wavelength (for example, 500nm) is made to penetrate, as shown in this drawing (b). In addition, as the excitation light source 70, you may use the extra-high pressure mercury lamp 701 as

shown in this drawing (d).

[0076] (5) Irradiate the excitation light (for example, wavelength of 450nm) from the excitation light source 70 at the capillary-tube plate CA, and observe a fluorescence image, after arranging the capillary-tube plate CA in such a nucleobase sequenator. The fluorescence image obtained by this observation is the same as that of drawing 5 (a), and can determine the array of ******-ed like the 1st example. That is, since the amount of extension of a primer here is proportional to the amount of fluorescence from a fluorochrome and a primer is not elongated in the direction of 3'edge from 5'edge, the existence [primer / ******-ed and] of hybridization and the joint position of ******-ed and a primer are known. In addition, it is as the primer in the column of the capillary-tube plate CA having also shown the array to drawing 2 as mentioned above.

[0077] That is, in [TGC], [CGA], [GAC], [ACG], and the [GAT] column, since the fluorescence of the amount 5 of luminescence, 2.5, and 3, 2 and 0 is observed, respectively, if these are arranged in order with few amounts of luminescence according to the algorithm of a picture drawn without lifting the brush from the paper, the base sequence elongated to a primer called TCGACGAT will be determined. And since ******-ed is a nucleic acid complementary to this, ******-ed can be identified [AGCTGCTA] (drawing 5 (b)). Moreover, it is also possible to determine the base sequence of long-chain ******-ed as shown in drawing 6 also by this method.

[0078] next, the inside of a column -- actually -- four kinds of fluorochrome-dNTP(s) (namely, -dATP, -dTTP, -dGTP, -dCTP) -- DNA polymerase -- as a catalyst -- introducing -- four kinds of fluorochrome-dNTP(s) -- abbreviation -- by adding to ******-ed simultaneously, hybridization arose and the following experiments confirmed whether the fluorescence (luminescence) proportional to the joint position of ******-ed and a primer would be observed

[0079] (Example 2 of an experiment) The fixed quantity of dNTP incorporated with an extension reaction was performed. When making DNA incorporate dNTP at the extension reaction by DNA polymerase, it uses for fluorochrome-dNTP instead of a part or all dNTP(s). When a part of dTTP and compatible fluorescein-12-dUTP (or rhodamine-4-dUTP) were used, the extension reaction occurred. In this case, it is necessary to fix the nucleic acid crossed in order to remove the unreacted fluorescence label dNTP by washing in a tube wall etc. Here, it washed by fixing in a magnetic bead as alternative operation.

[0080] (Material and method)

Reagent streptoavidin fixed MAG bead (Dynabeads M-280)

The one 224 base length chain DNA with which 3'end was biotin-ized (multi-cloning site of plus DOMIDO)

Reverse primer KS primer DNA polymerase (AmpliTaqTMDNA polymerase)

dNTP buffer solution (dATP-dGTP-dCTP every 25microM, dTTP8microM, fluorescein-12-dUTP(or rhodamine-4-dUTP)2microM, and 10mM-tris and a base (pH 8.3), 1.5 mM-MgCl2, 50 mM-KCl, 0.01% gelatin)

Penetrant remover (10mM-tris and a hydrochloric acid (pH 7.5), 2 mM-EDTA, 1 M-NaCl)

Thermostat spectrophotofluorometer experiment operation **** for the test tube

electrode holders PCR with 80% formamide solution laboratory instrument magnet and two test tubes (a, b) are prepared, and the following were added to each.
[0081] a) The dNTP buffer solution (50microl), the 1 chain DNA (one picomole), DNA polymerase (one unit), a reverse primer (two picomoles)
b) The dNTP buffer solution (50microl), the 1 chain DNA (one picomole), DNA polymerase (one unit), KS primer (two picomoles)
Next, each was incubated in order of 95 degrees C (30 seconds), 41 degrees C (30 seconds), and 72 degree-C(5 minutes) **. Then, it kept at 4 degrees C immediately.
[0082] After an appropriate time, a magnetic bead (50microl) is added and agitated and it sets to an electrode holder with a magnet. As a magnetic bead was not contacted, top ** was thrown away with the pipet, it washed by the penetrant remover and distilled water washed the magnetic bead once twice. And the magnetic bead was suspended in 80% formamide solution of 500microl, and the complementary strand which incubated for 3 minutes and was elongated at 95 degrees C was eluted.
[0083] Next, it sets to an electrode holder with a magnet again, and a fluorescence spectrum is shortly measured for the supernatant containing the extension complementary strand for the cuvette of a spectrophotometer. In the case of fluorescein, in the case of 488nm and the rhodamine, excitation light used 540nm. The fluorescence spectrum of DNA which incorporated fluorescein and the rhodamine is shown in drawing 9 . Moreover, the fluorescence intensity in 520nm and 580nm was used for measurement of the length of the extension reaction using each fluorochrome. Moreover, as a result of measuring such fluorescence intensity (the amount of luminescence), when the fluorochrome of fluorescein was used, the fluorescence intensity of 3.35 (a. u.) was observed with the test tube (a) containing the reverse primer, and the fluorescence intensity of 1.71 (a. u.) was observed with the test tube (b) containing KS primer. Moreover, when the fluorochrome of a rhodamine was used, the fluorescence intensity of 10.10 (a. u.) was observed with the test tube (a) containing the reverse primer, and the fluorescence intensity of 5.26 (a. u.) was observed with the test tube (b) containing KS primer.
[0084] Each fluorescence intensity ratio of two extension reactions became about 1.9. Although the ratios of the actually elongated length (the number of bases) are 208 / 127= 1.63, when a number of fluorochrome-dUTP (namely, position of T) of ratios which should be incorporated are calculated, it turns out that it is set to 1.96 and has become the actual measurement of fluorescence intensity, and a very near value.
[0085] That is, the ratio of the length which reverse and KS primer elongate by hybridization with the above-mentioned ******-ed can be determined from the grade fluorescence intensity which is also a time of using one kind of fluorochrome-dNTP, and since the kind of primer combined with the joint position of a primer and ******-ed became clear, when using the algorithm of a picture drawn without lifting the brush from the paper, thereby, the base sequence of ******-ed was able to be determined like the 1st example. Moreover, since it is possible to measure precisely not only the kind of primer but the joint position of this and ******-ed of mold by raising measurement precision further, two or more oiler paths of the picture drawn without lifting the brush from the paper which has not been determined since a joint position was not able to be pinpointed are able to determine the array of a certain nucleobase.

[0086] In addition, this invention is not limited to the above-mentioned example. That is, although the length which measured the amount of the pyrophosphoric acid which separated (A'), or observed luminescence from a fluorescence (I') label, and was elongated was found in the above 1st and the 2nd example, the following is mentioned as a method of finding this elongated length.
[0087] (a) Measure the amount of the degradation product which separates with an extension reaction.
[0088] (b) Measure the amount of the base incorporated in a nucleic acid with an extension reaction.
[0089] (c) Observe the partial length of a direct double chain with an electron microscope, a tunneling microscope, etc. which have the resolution of atomic level for ******-ed after an extension reaction.
[0090] (d) It is also possible to apply to gel electrophoresis like the method which separates the product (complementary strand nucleic acid) of an extension reaction itself, and is put in practical use now, and to find length.
[0091] Moreover, since it was easy to arrange a primer in the shape of a matrix, although the rectangle-like capillary-tube plate CA was used in the above 1st and the 2nd example, this may use a circular thing.
[0092]
[Effect of the Invention] According to the nucleobase sequencing method which starts this invention as above, first, hybridization of a primer and the ******-ed is carried out, and these complex is formed according to the 1st process, next the 2nd process performs the extension reaction from 5' of a primer to the direction of 3' by using ******-ed as mold, and the 3rd process detects the amount of extension of a primer. Since the kind of primer combined in the joint position, the amount of extension of each primer, i.e., the primer, arranged to two or more fields for a short time, of ******-ed and each field by this can be known, if the base sequence of a primer is analyzed using the so-called algorithm of a picture drawn without lifting the brush from the paper, it is possible to determine the base sequence of ******-ed.
[0093] Furthermore, since it is possible to measure precisely not only the kind of primer but the joint position of this and ******-ed of mold by raising measurement precision further, two or more oiler paths of the picture drawn without lifting the brush from the paper which has not been determined since a joint position was not able to be pinpointed are able to determine the array of a certain nucleobase. Moreover, such two or more fields' being specified by the column of the capillary-tube plate which consists of material made from glass or silicon, then primers are not mixed.
[0094] It is good for the method of detection of such an amount of extension also as carrying out by detecting the amount of luminescence accompanying the luciferase reaction of ATP resulting from the pyrophosphoric acid isolated from two or more kinds of nucleotides, or the nucleotide analog with the extension reaction, and It is good also as performing the extension reaction of the primer which added the deoxyribonucleotide 3 phosphoric acid (dNTP) which carried out the fluorescence label as a nucleotide analog, and used ******-ed as mold, and carrying out by

detecting the future amount of luminescence. Thereby, since the amount of luminescence is proportional to the amount of extension, it can ask for the joint position of the kind of primer, the united primer, and united ******-ed.

[Translation done.]

* NOTICES *

DESCRIPTION OF DRAWINGS

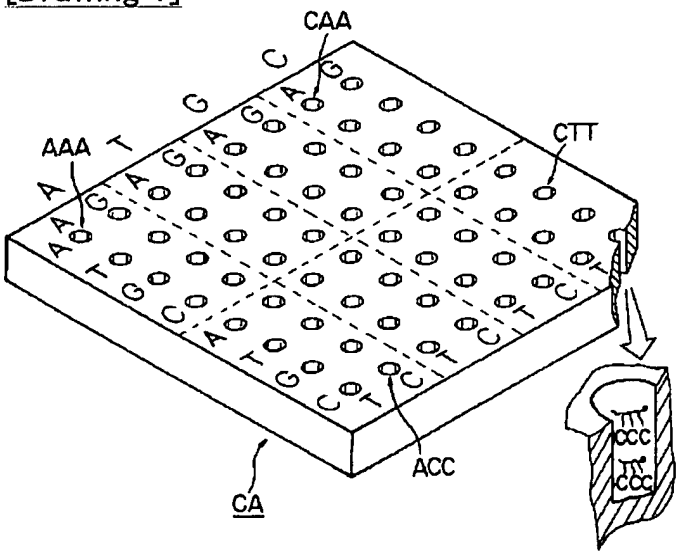
[Brief Description of the Drawings]
[Drawing 1] It is the perspective diagram of the capillary-tube plate used in the example of this invention.
[Drawing 2] It is array explanatory drawing showing the array of the primer within the capillary-tube plate of drawing 1 .
[Drawing 3] It is explanatory drawing explaining the procedure of the 1st example of this invention.
[Drawing 4] It is the tropia block diagram of the nucleobase sequenator used in the 1st example.
[Drawing 5] It is explanatory drawing for explaining identification of luminescence in the capillary-tube plate of drawing 1 , and ******-ed.
[Drawing 6] It is explanatory drawing for explaining identification of long-chain ******-ed.
[Drawing 7] It is explanatory drawing explaining the procedure of the 2nd example of this invention.
[Drawing 8] It is the tropia block diagram of the nucleobase sequenator used in the 2nd example.
[Drawing 9] It is drawing showing the relation between the luminescence intensity from DNA, and wavelength which was measured in the 2nd example, and which carried out the fluorescence label.
[Drawing 10] It is explanatory drawing for explaining the conventional SBH method.
[Description of Notations]
CA [-- A CCD camera, 20 / -- A microscope, 30 / -- An image processing system, 40 / -- A computer, 50 / -- An excitation light-transmission filter, 60 / -- A fluorescence transparency filter, 70 / -- The excitation light source, 701 / -- Extra-high pressure mercury lamp.] -- A capillary-tube plate, DB, DB1 -- A black box, 10

[Translation done.]

* NOTICES *

DRAWINGS

[Drawing 1]

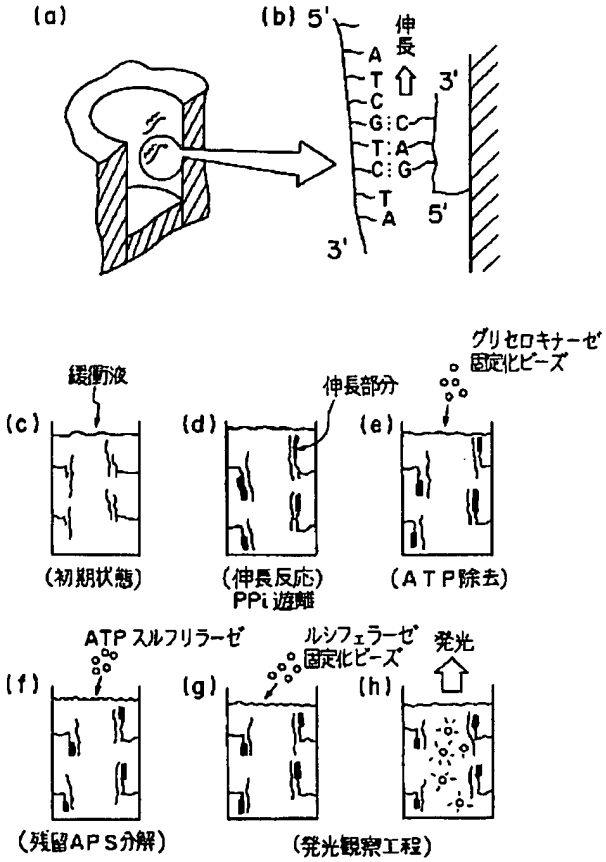


[Drawing 2]

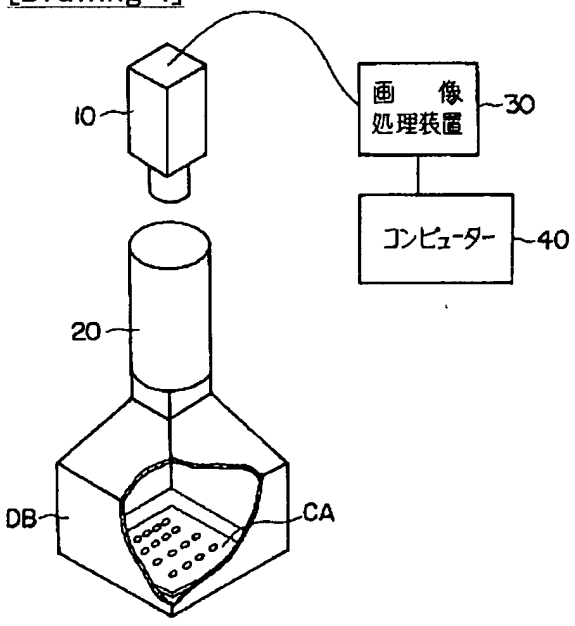
j s f	j s f	j s f	j s f	j s f	j s f	j s f	j s f
AAA	AGA	TAA	TGA	GAA	GGA	CAA	CGA
AAT	AGT	TAT	TGT	GAT	GGT	CAT	CGT
AAG	AGG	TAG	TGG	GAG	GGG	CAG	CGG
AAC	AGC	TAC	TGC	GAC	GGC	CAC	CGC
ATA	ACA	TTA	TCA	GTA	GCA	CTA	CCA
ATT	ACT	TTT	TCT	GTT	GCT	CTT	CCT
ATG	ACG	TTG	TCG	GTG	GCG	CTG	CCG
ATC	ACC	TTC	TCC	GTC	GCC	CTC	CCC

キャピラリープレート内のプライマーの配置

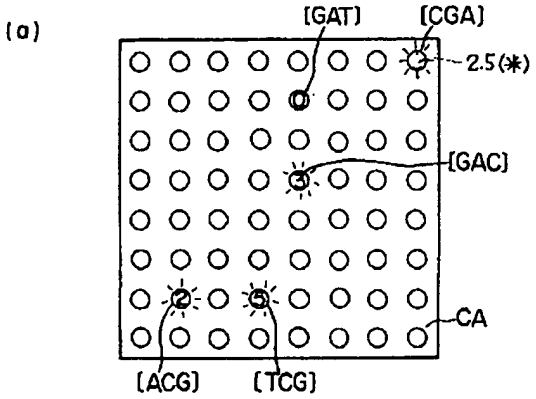
[Drawing 3]

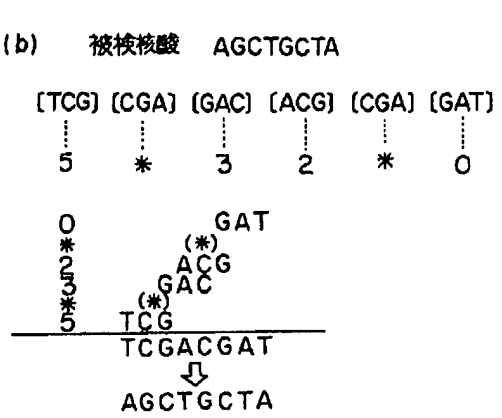


[Drawing 4]

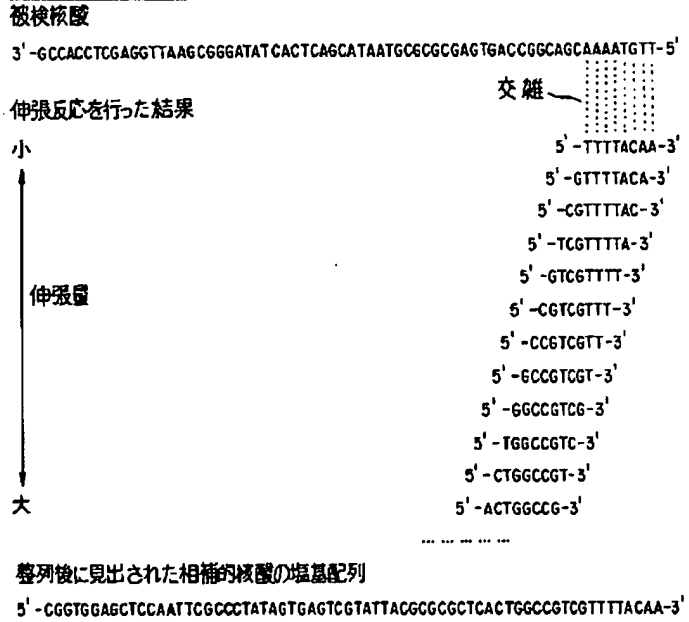


[Drawing 5]

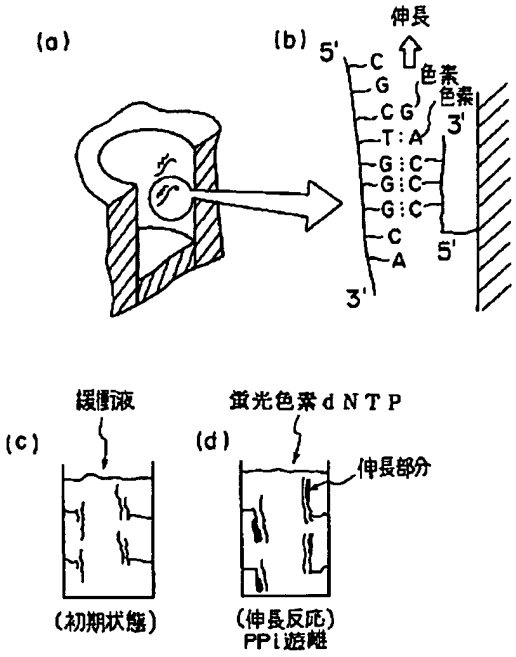




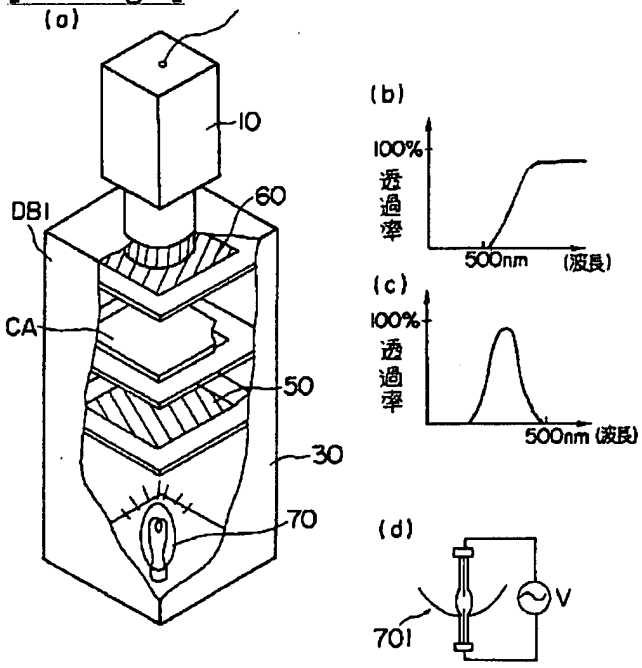
[Drawing 6]



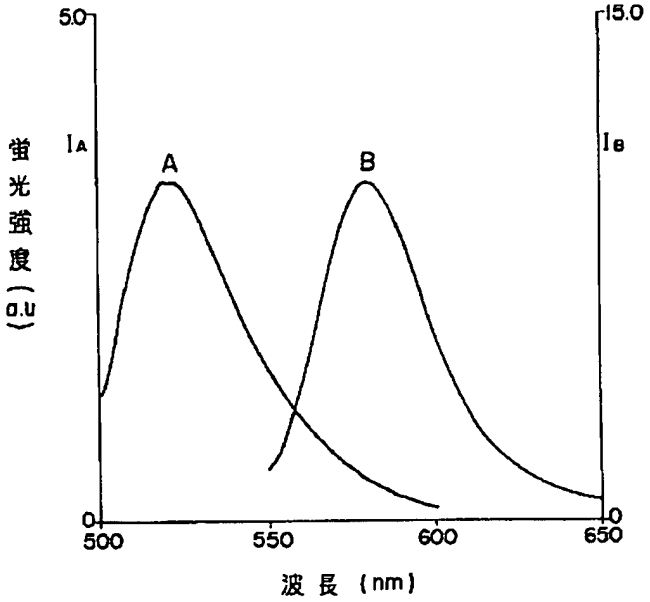
[Drawing 7]



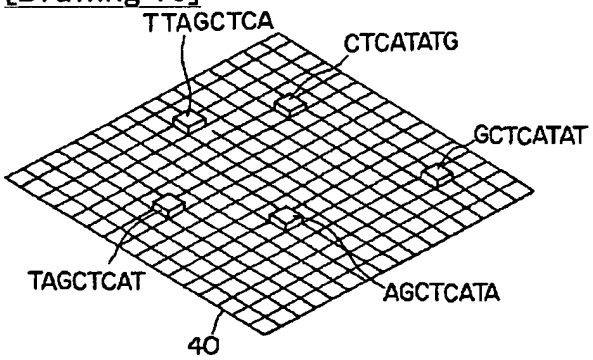
[Drawing 8]



[Drawing 9]



[Drawing 10]



TTAGCTCA
TAGCTCAT
AGCTCATA
GCTCATAT
CTCATATG

TTAGCTCATATG
(被模板酸)→AATCGAGTAGAC

[Translation done.]